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β-Dystrobrevin, a New Member of the Dystrophin Family

IDENTIFICATION, CLONING, AND PROTEIN ASSOCIATIONS*

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Dystrophin, the protein disrupted in Duchenne muscular dystrophy, is one of several related proteins that are key components of the submembrane cytoskeleton. Three dystrophin-related proteins (utrophin, dystrophin-related protein-2 (DRP2), and dystrobrevin) have been described. Here, we identify a human gene on chromosome 2p22–23 that encodes a novel protein, β-dystrobrevin, with significant homology to the other known dystrobrevin (now termed α-dystrobrevin). Sequence alignments including this second dystrobrevin strongly support the concept that two distinct subfamilies exist within the dystrophin family, one composed of dystrophin, utrophin, and DRP2 and the other composed of α- and β-dystrobrevin. The possibility that members of each subfamily form distinct protein complexes was examined by immunopurifying dystrobrevins and dystrophin. A β-dystrobrevin antibody recognized a protein of the predicted size (71 kDa) that copurified with the dystrophin short form, Dp71. Thus, like α-dystrobrevin, β-dystrobrevin is likely to associate directly with dystrophin. α- and β-dystrobrevins failed to copurify with each other, however. These results suggest that members of the dystrobrevin subfamily form heterotypic associations with dystrophin and raise the possibility that pairing of a particular dystrobrevin with dystrophin may be regulated, thereby providing a mechanism for assembly of distinct submembrane protein complexes.

Positional cloning of the gene altered in Duchenne muscular dystrophy resulted in the description of dystrophin, a key component of the membrane skeleton (reviewed in Ref. 1 and 2). Dystrophin is a large protein of 427 kDa with an NH_2-terminal actin binding domain, a central rod region composed of spectrin-like repeats, and cysteine-rich (CR)[1] and carboxyl-terminal (CT) domains. In skeletal muscle, dystrophin binds cortical actin via its NH_2-terminal region (3) and several spectrin-like repeats (4). The CR and CT domains of dystrophin bind a complex of membrane proteins collectively termed the dystrophin-associated proteins (DAPs) (reviewed in Ref. 5). Several DAPs are transmembrane glycoproteins, including β-dystroglycan, which binds directly to dystrophin and α-dystroglycan, an extracellular laminin binding protein. Thus, as a whole, the dystrophin glycoprotein complex is thought to link the extracellular matrix to the cytoskeleton, thereby stabilizing the sarcolemma during repeated cycles of muscle contraction and relaxation. Consistent with this structural role, mutations in dystrophin and in certain DAPs are the primary defects leading to Duchenne muscular dystrophy and limb girdle muscular dystrophies, respectively (1, 6).

Dystrophin is only one member of a growing family of related proteins. In part, the complexity of this family results from the production of several products from the dystrophin gene. The use of alternative promoters within the dystrophin gene yields proteins that lack the amino-terminal actin binding domain and many or all of the spectrin-like repeats (2). One form, Dp71, is composed of only the CR and CT domains. Additional complexity stems from the finding that several proteins related to dystrophin are encoded by separate genes. Of these, utrophin is most closely related to dystrophin. The basic domain structure of utrophin is quite similar to dystrophin, although it has two fewer spectrin-like repeats (reviewed in Ref.7), and it is thought to bind a similar complex of proteins (8). In contrast to dystrophin, which is expressed primarily in skeletal muscle, utrophin is broadly distributed and thus may serve a function similar to that of dystrophin in nonmuscle cell types (9, 10). Dystrophin-related protein-2 (DRP-2) is closely related to dystrophin and utrophin but is encoded by a separate gene and contains only two spectrin-like repeats in addition to the CR and CT domains (11).

A fourth, more distant member of the dystrophin family is dystrobrevin, first described as a tyrosine-phosphorylated 87-kDa protein in *Torpedo* electric organ (12, 13). *Torpedo* dystrobrevin is composed of CR and CT domains with modest homology to dystrophin and a unique COOH-terminal tail containing putative tyrosine phosphorylation sites. It has no spectrin-like repeats. Several forms of dystrobrevin, generated by alternative splicing, have been identified in mammalian tissues (14, 15). Three variable regions within the coding sequence undergo tissue-specific splicing, generating forms that are specifically expressed in muscle. In addition, splicing produces a form of dystrobrevin that lacks the unique tail. Dystrobrevin is found in dystrophin preparations and probably interacts directly with dystrophin (17).[2] Thus, dystrobrevin is not only a dystrophin-related protein but also a dystrophin-associated protein.

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[1] The abbreviations used are: CR, cysteine-rich; CT, carboxyl-terminal; Ab, polyclonal antibody; CRCT, cysteine-rich COOH-terminal region of dystrophin; DAP, dystrophin-associated protein; DRP, dystrophin-related protein; DUR, dystrobrevin-unique region; EST, expressed sequence tag; mAb, monoclonal antibody; contig, group of overlapping clones; kb, kilobases; vr, variable regions.

[2] Sadoulet-Puccio, H. M., Rajala, M. and Kunkel, L. M., *Proc. Natl. Acad. Sci.*, in press.

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The function of dystrophin, utrophin, and their associated proteins is probably not limited to the structural role proposed for the dystrophin complex in skeletal muscle. Dystrophin expressed in the central nervous system is highly concentrated at the postsynaptic membrane (18), where its loss is associated with altered synaptic transmission (19) and, in some cases, nonprogressive cognitive impairment (1). At the neuromuscular synapse, utrophin is colocalized with nicotinic acetylcholine receptors at the crests of the postjunctional folds (20), whereas dystrophin is most concentrated in the bottoms of the folds along with sodium channels (20–22). Thus, even within this single synapse, dystrophin and utrophin are likely to serve quite different roles. Possible functions for these complexes at this synapse include involvement in agrin-induced clustering and anchoring of acetylcholine receptors (23–26) or sodium channels (27, 28) at the postsynaptic membrane. However, mice lacking both utrophin and dystrophin show surprisingly subtle abnormalities in the neuromuscular junction (29, 30). Thus, although each dystrophin-related protein may be involved in the assembly of a particular membrane specialization, definitive tests for this proposal have yet to be done.

The function of dystrophin and related proteins likely depends on the proteins associated with them. One group of DAPs, the syntrophins, are a multigene family composed of three closely related isoforms (α1, β1, and β2) (31–33). Syntrophins are thought to be modular adaptor proteins that bind directly to dystrophin family members and in turn recruit signaling proteins such as neuronal nitric oxide synthase (34) and voltage-gated sodium channels to the membrane (27). In an attempt to expand the repertoire of the dystrophin-related protein complex, we sought to identify new members of the dystrophin family that bind syntrophins. Using the syntrophin binding motif identified in dystrophin and dystrobrevin (see Ref. 35) as a probe to search sequence data bases, we discovered a new dystrophin-related protein, β-dystrobrevin. The same protein was discovered independently by data base searches with the entire α-dystrobrevin sequence. Sequence comparisons with the previously identified α-dystrobrevin support the idea that the dystrobrevins comprise a distinct subfamily within the dystrophin family. Since β-dystrobrevin, like α-dystrobrevin, associates with dystrophin, we propose a model in which dystrobrevin subfamily members form heterotypic associations with dystrophin subfamily members.

EXPERIMENTAL PROCEDURES

Isolation of β-Dystrobrevin cDNA—Both strands of the clones containing the sequences reported in expressed sequence tags (ESTs) R15062 (clone 29483), R38788 (clone 24544), H16100 (clone 48688), H26850 (clone 158283), and H42052 (clone 182424) (Genome Systems, St. Louis, MO) were sequenced and found to encode most of β-dystrobrevin, lacking only the 5′ coding region.

The remaining coding region was obtained by amplification of adapter-ligated human liver cDNA (CLONTECH, Palo Alto, CA) using the Expand high fidelity polymerase chain reaction system (Boehringer Mannheim). Polymerase chain reaction products were cloned into Bluescript II (Stratagene, La Jolla, CA), and multiple clones were sequenced. A human adult brain cDNA library (CLONTECH) was screened with a 650-base pair probe generated by polymerase chain reaction of EST R15062 with the primer pair 5′-CAGAAGCAGGAAACGTGACTC and 5′-CTCCTCTGCTAACCTCTGTC. Three overlapping clones corresponding to the 5′ coding region were sequenced.

All sequences were determined using a model 373A DNA sequencer (Applied Biosystems, Foster City, CA) by the University of North Carolina, Chapel Hill, automated DNA sequencing facility or the MRRC Automated Sequencing Core Facility. Sequences were analyzed with the aid of a DNAStar Lasergene computer software package (Madison, WI).

Chromosomal Localization of the β-Dystrobrevin Gene—Fluorescence *in situ* hybridization analysis was done according to standard protocols (36–38). Three genomic phage clones covering the first coding exon and flanking introns (HGL2, HGL10, HGL19) and two genomic phages covering the region homologous to exon 18 in α-dystrobrevin (39) and the flanking introns (HGL9, HGL18) were isolated from a human genomic library. Phage DNA was isolated by a cesium gradient and treated with RNase. DNA from phage clones HGL2, HGL10, and HGL19 were pooled as were phages HGL9 and HGL18. 1 μg of phage DNA pool was labeled with digoxigenin-11-dUTP by nick translation, as described previously (36, 37). Slides were examined on a Zeiss Axiophot microscope. Visual inspection of the slides through a triple band-pass filter (Omega, Brattleboro, VT) allowed detection of the DNA hybridization signal over the 4′,6′-diamidino-2-phenylindole counterstained nuclei.

Yeast artificial chromosome analysis was done for the 5′ and 3′ ends of the gene by screening a pooled human yeast artificial chromosome array (Research Genetics Inc., Huntsville, AL). Primer pairs from the first coding exon and its upstream intron (forward primer 5′-AGTTAGAAGTAAGTCAGCCCC-3′ and reverse primer 5′-AGCACGCATTTCTATGAACAGC-3′), giving a 400-base pair product and from exon 18 and its downstream intron (forward primer 5′-CACCACATACATCGCCCACC-3′ and reverse primer 5′-GGAGGAGACAAAGCCCAAAGGACA-3′), giving a 330-base pair product, were used. Polymerase chain reactions were performed with a 0.01 volume of [α-^{32}P]dCTP or [α-^{32}P]dGTP to label the product, which was visualized by being run on an 8% acrylamide gel and exposed to film.

Northern Blot Analysis—A human multiple tissue Northern blot (CLONTECH) was probed according to the manufacturer's protocol using a random primed ^{32}P-labeled probe containing the entire coding region of β-dystrobrevin.

Antibodies—To prepare polyclonal antibodies (Abs), synthetic dystrobrevin peptides with a terminal cysteine were coupled to keyhole limpet hemocyanin and injected into rabbits according to standard methods (Covance Inc., Denver, PA). Ab βDB344 was prepared against the peptide DTMVSHMSSGVPTPTKSVLDSPS-COOH corresponding to amino acids 344–366 of β-dystrobrevin, which are highly divergent from α-dystrobrevin. Ab αDB2 was prepared against a peptide corresponding to the unique COOH-terminal 10 residues of α-dystrobrevin-2 (COOH-GVSYVPYCRS) (14, 15). Ab DB433 was prepared previously against a peptide corresponding to residues 433–451 of murine α-dystrobrevin (14). Since this region is identical between the human α- and β-dystrobrevins, Ab DB433 is likely to recognize products of both dystrobrevin genes. Antibodies were affinity purified from serum using peptide coupled to Affi-Gel 10 or 15 (Bio-Rad).

All other antibodies have been previously described. The syntrophin monoclonal antibody (mAb) SYN1351 (40) recognizes all three known syntrophin isoforms (41). mAb 13H1 (a gift of J. B. Cohen, Harvard Medical School) was raised against *Torpedo* dystrobrevin (12). The anti-dystrophin mAb MANDRA-1 was purchased from Sigma. Ab DYS3669 was prepared against COOH-terminal 10 amino acids of mouse dystrophin (41).

Immunoaffinity Purification and Immunoblotting of Protein Complexes—Immunoaffinity purification of protein complexes from rat tissues (Pel-Freez, Rogers, AZ) was done as described previously (9). Immunoblotting was performed with a primary antibody concentration of 30 nM as described previously (40).

RESULTS

Isolation of β-Dystrobrevin cDNAs—In vitro binding studies have identified a region in the CT domain of dystrophin, utrophin, and dystrobrevin that binds syntrophins (17, 42–45). To identify new syntrophin binding proteins, we searched protein data bases with a sequence corresponding to the consensus syntrophin binding motif (reviewed in Ref. 35). Of the many ESTs identified, several encode a protein that is homologous to, but distinct from, known dystrophin-related proteins. Subcloning and complete sequencing of these ESTs confirmed that they encode a protein homologous to the carboxyl-terminal half of dystrobrevin. The additional sequence 5′ of the EST was obtained by both 5′ rapid amplification of cDNA ends and isolation of additional clones from a human brain cDNA library (Fig. 1*A*). The combined cDNAs (2.4 kilobase pairs) contain an 1,881-nucleotide open reading frame with the position of the first ATG closely corresponding to that of the first ATG in the previously described dystrobrevin (Fig. 1*B*) (13–15). The second ATG is in the appropriate consensus translation start sequence (46) and, if utilized, would produce a protein lacking the first 11

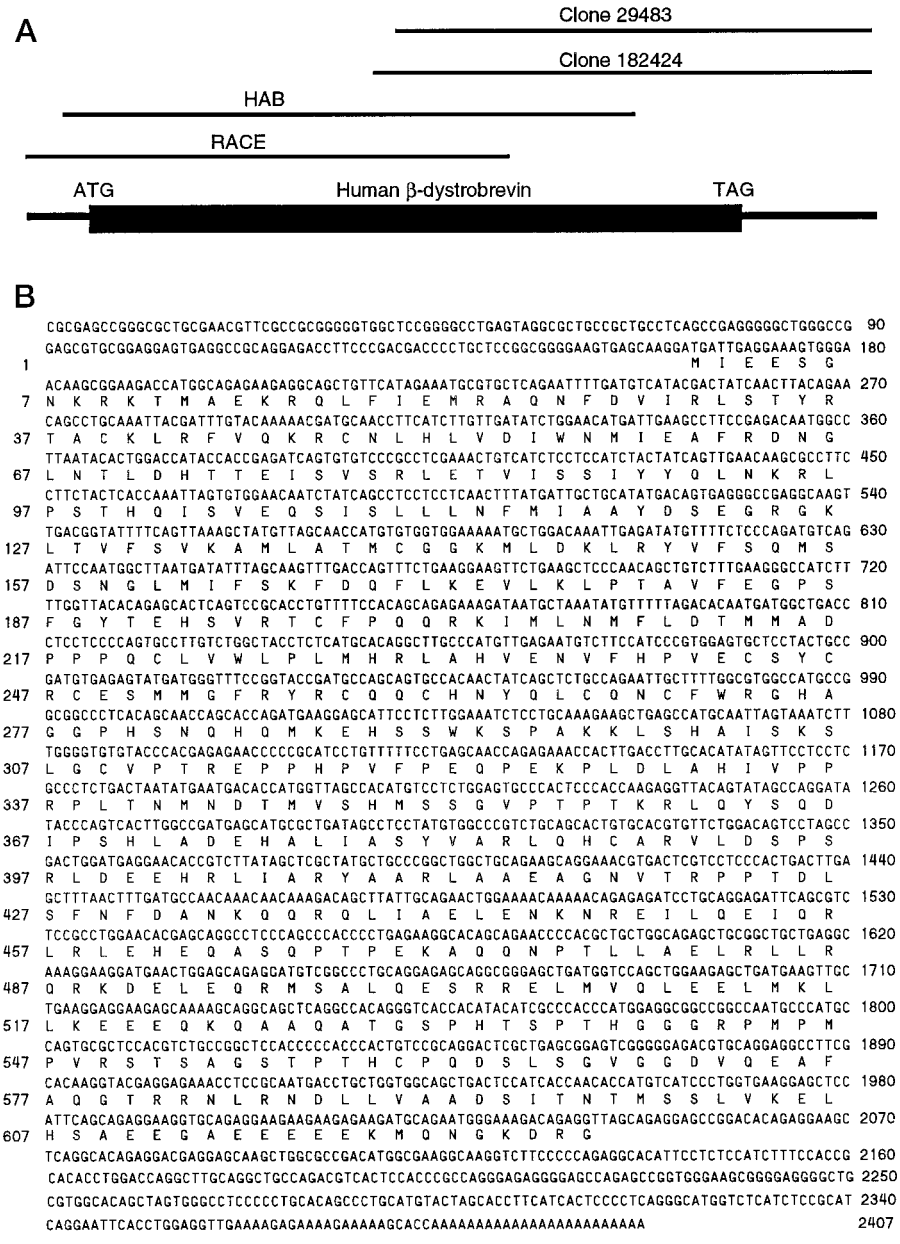

FIG. 1. **β-dystrobrevin, a new DRP gene.** *A*, a schematic of the β-dystrobrevin cDNA cloning strategy. *B*, the nucleotide and deduced amino acid sequence of human β-dystrobrevin resulting from a merge of clones 29483 and 182424, 5′ rapid amplification of cDNA ends products, and clone HAB are shown. The positions of the nucleotides are shown to the *right*, and amino acids are shown to the *left*.

amino acids. However, it seems likely that the sequence upstream of the second ATG is translated, since the protein sequence is highly conserved with the previously described dystrobrevins (see below) (13–15). The encoded protein of 627 amino acids (Fig. 1*B*) has a calculated molecular mass of 71,362.5 Da and a predicted isoelectric point of 7.88. The entire amino acid sequence is 68% identical with human dystrobrevin but much less related to dystrophin and utrophin (see below). Thus, we have named this newly identified protein β-dystrobrevin and suggest that the previously identified dystrobrevin (14, 15) be called α-dystrobrevin.

Amino Acid Sequence Comparison of α- and β-Dystrobrevin—α- and β-dystrobrevins have highly similar NH_2-terminal regions, with conservation extending throughout their sequences. The most noticeable differences occur in the COOH-terminal truncations. The largest of the dystrobrevin proteins is α-dystrobrevin-1, which is composed of 557 amino acids homologous to the CRCT region of dystrophin plus an additional 188 amino acids with no homology to other proteins. This dystrobrevin-unique region (DUR) contains sites for tyrosine phosphorylation near the COOH terminus (13), a feature consistent with the observation that *Torpedo* dystrobrevin is a tyrosine-phosphorylated protein (13). In α-dystrobrevin-2, the entire DUR is removed by alternative splicing (14, 15). β-Dystrobrevin contains only the proximal half of the DUR, and as a consequence, lacks the tyrosine phosphorylation sites.

The work of several laboratories has defined a core region of approximately 16 amino acids as the site in dystrobrevin that binds syntrophins (Refs. 17 and 42–45; reviewed in Ref. 35). The sequence of this motif is identical in all mammalian dystrobrevins (α-dystrobrevins from human and mouse and the human β-dystrobrevin described here) and in *Torpedo* dystrobrevin (13–15) (see also Fig. 5*A*). Thus, it seems likely that each of the dystrobrevins binds syntrophins.

Variable Regions in β-Dystrobrevin cDNAs—In comparison with the full-length β-dystrobrevin coding sequence, several classes of ESTs could be identified, based on small in-frame deletions in the coding region. These variable regions in β-dystrobrevin clones were compared with the previously described variable regions (vr1–vr3) in α-dystrobrevin (Fig. 2*A*). In α-dystrobrevin, vr1 denotes alternative splicing of exon 9, which encodes three amino acids. The corresponding three

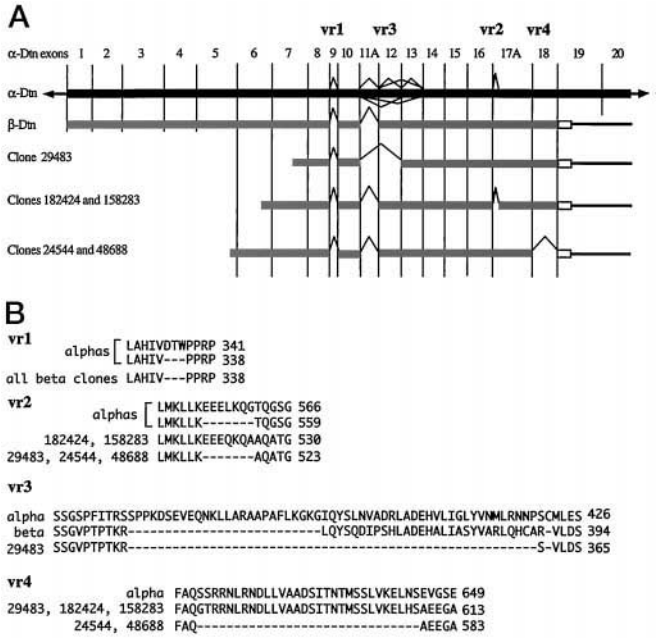

FIG. 2. **Comparison of the variable regions in α- and β-dystrobrevins.** *A*, a schematic comparing the variable regions in β-dystrobrevin (*β-Dtn*) with the known splicing and exon structure of human α-dystrobrevin (*α-Dtn*) (39). *Wide lines* indicate coding regions, whereas *thinner lines* denote 5′- and 3′-untranslated regions. β-Dystrobrevin sequences homologous to α-dystrobrevin are shown schematically in gray, whereas nonconserved regions are shown in *white*. Note that each of the variable regions in β-dystrobrevin is bounded by exons borders identified in α-dystrobrevin, suggesting conservation of these intron-exon borders in β-dystrobrevin. *B*, the predicted amino acid sequences of β-dystrobrevin clones are aligned with the vr identified in α-dystrobrevin (14, 15). β-Dystrobrevin sequences are indicated by clone number with the amino acid positions shown as if clones extended to the COOH terminus. In vr1, a nine-base pair exon is spliced in certain α-dystrobrevins. These nine nucleotides were absent in all β-dystrobrevin clones examined. In vr2, both the full-length and spliced forms of α-dystrobrevin are highly conserved in β-dystrobrevin. The region corresponding to vr3, which is subject to extensive splicing in α-dystrobrevin, revealed two variants in β-dystrobrevin. In full-length β-dystrobrevin, amino acid 390 is an Arg (*R*), whereas in clone 29483, the corresponding residue is a Ser (*S*). The codon for Arg-390 (R390) occurs at the position corresponding to the end of α-dystrobrevin exon 13 and is changed by the presence or absence of that exon. All β-dystrobrevin clones lacked either 27 or 56 amino acids in vr3. An additional variable region in β-dystrobrevin (termed vr4) was identified in selected clones. Note that both the spliced region (indicated by *dashes*) and selected sequences surrounding the splice site are shown.

amino acids are absent from all β-dystrobrevin clones examined. Additional β-dystrobrevin clones were identified that corresponded to α-dystrobrevin splice forms in which vr2 and vr3 are absent. Finally, a previously undescribed variable region (vr4) was identified in multiple β-dystrobrevin clones in the region encoding the DUR.

Each of the variable regions in β-dystrobrevin corresponds precisely to exon boundaries in the α-dystrobrevin gene (Fig. 2, *A* and *B*). This suggests that the multiple β-dystrobrevin coding regions identified at vr2, vr3, and vr4 are generated by alternative splicing similar to α-dystrobrevin. In vr1 and part of vr3, it is unclear if the region corresponding to exons 9 or 11A in α-dystrobrevin is deleted from the β-dystrobrevin gene or if this form is expressed at low levels. Further characterization of the β-dystrobrevin gene will be required to determine if additional β-dystrobrevin coding regions exist.

Tissue-specific Expression of β-Dystrobrevin mRNA—Northern blot analysis with a hybridization probe corresponding to the coding region of β-dystrobrevin revealed that all tissues examined express detectable β-dystrobrevin transcripts (Fig. 3). A major

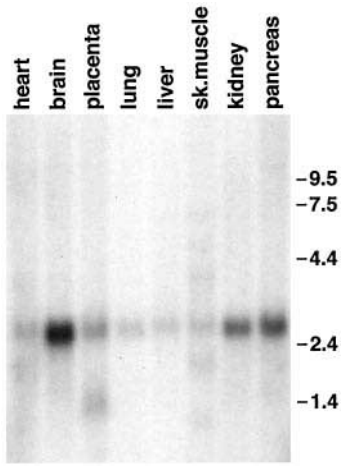

FIG. 3. **mRNA tissue distribution of β-dystrobrevin.** A Northern blot of mRNA isolated from human tissues was probed with cDNA containing the coding region of β-dystrobrevin. Markers are in kb. *sk*., skeletal.

transcript of ~2.5 kb is highly expressed in brain, kidney, and pancreas. In addition, low levels of additional transcripts of 1.0, 2.0, 3.5, 5.0, and 7.0 kb were found in skeletal muscle. Heart and placenta expressed additional β-dystrobrevin transcripts of 2.0 and 1.4 kb, respectively.

Chromosomal Localization of β-Dystrobrevin Gene—The β-dystrobrevin gene was localized to chromosome 2p22–23 by two independent methods. Human genomic clones were isolated and used for fluorescence *in situ* hybridization to human chromosomes (47). Visual analysis of multiple metaphase spreads hybridized either with clones of the 5′ end of the gene or the 3′ end showed specific hybridization to the short arm of chromosome 2 in the region 2p22–23 (Fig. 4). Two primer pairs from the 5′ and 3′ coding regions of the gene were used to screen a pooled human yeast artificial chromosome array, identifying 943f12 with the 5′ pair and 953d6 with the 3′ pair. The overlapping yeast artificial chromosomes are part of the singly linked contig WC2.2 and contain the marker D2S2144 (a CA dinucleotide repeat), which is localized at a genetic distance of 49.5 centimorgans from the apter, consistent with the localization by fluorescence *in situ* hybridization to 2p22–23. The α-dystrobrevin gene has been localized to human chromosome 18q12 (39), confirming that the two dystrobrevins are encoded by distinct genes. Neither has yet been shown to correlate with a mapped human disease.

Sequence Comparison of Dystrobrevins with Dystrophin Family Members—The sequence of β-dystrobrevin was compared with the sequences of human dystrophin and the three known dystrophin-related proteins (Fig. 5). All five gene products share ~30% amino acid identity in the region corresponding to the CRCT region of dystrophin (Fig. 5*A*, residues *shaded black*). Among the divergent residues, however, the two dystrobrevins are highly similar to each other but not to dystrophin, utrophin, or DRP2 (Fig. 5*A*, residues *shaded gray*). Conversely, the corresponding residues in dystrophin, utrophin, and DRP2 are highly similar to each other but not to the dystrobrevins (Fig. 5*A*, residues in *light box*). In the region shown, pair-wise comparisons reveal that the two dystrobrevins have 76% identity, and the dystrophins (dystrophin, utrophin, and DRP2) have 59–72% identity. In contrast, comparisons between dystrobrevins and dystrophins reveal only 26–30% identity (Fig. 5*B*). Thus, even in the most highly conserved region, the dystrophin family appears to be composed of a dystrophin subfamily and a dystrobrevin subfamily (shown schematically in Fig. 5*C*).

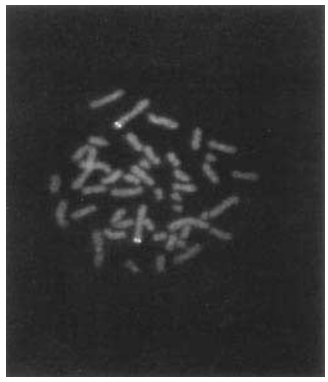

FIG. 4. **β-Dystrobrevin chromosomal localization.** Metaphase spreads were examined to localize β-dystrobrevin to the short arm of chromosome 2 between 2p22 and 2p23.

Although all the members of the dystrophin family have apparently similar domains, certain key residues within these domains differ between the two subfamilies. The protein domains originally found in the CRCT region of dystrophin include a WW domain (48), a pair of putative EF hands (49), a putative zinc finger (50), a syntrophin binding region (reviewed in Ref. 35), and two tandem leucine zipper coiled-coils (51) (Fig. 5*A*, *bold boxes*). The sequence characteristics of WW domains (W, WX_2P) are strictly conserved in dystrophin, utrophin, and DRP2, whereas in α- and β-dystrobrevins, the WW domain is absent (Fig. 5*A*). In the putative zinc finger region, dystrophin, utrophin, and DRP2 contain only two cysteine pairs (CX_2C), whereas α- and β-dystrobrevins both have a third cysteine pair. The latter thus have a characteristic ZZ finger sequence (CX_2CX_5CX_2C) (50) (Fig. 5*A*). In the tandem coiled-coils, the two heptad repeats of leucines are highly conserved in all five dystrophin family members, but the intervening nonleucine residues are highly specific for each subfamily. Thus, within the identified protein domains, key residues differ in a pattern consistent with two separate subfamilies.

β-Dystrobrevin Protein Expression and Associations with the Dystrophin Complex—To examine the expression and associations of α- and β-dystrobrevins, we used a monoclonal antibody that recognizes both α- and β-dystrobrevins (mAb DB13H1, referred to here as pan-Db; Ref. 12) and also prepared antibodies using isoform-specific dystrobrevin peptides. Ab αDB2 was made against the COOH-terminal 10 amino acids of α-dystrobrevin-2, the ~60-kDa isoform lacking the DUR. This sequence is absent from β-dystrobrevin. Ab βDB344 was prepared to a region of β-dystrobrevin that is highly divergent from the α-dystrobrevins (see Fig. 5*A*). Each antibody was used to immunoaffinity purify dystrobrevin complexes from rat brain, a rich source of both α- and β-dystrobrevins. The isolated complexes were then analyzed by immunoblotting. The β-dystrobrevin antibody (Ab βDB344) recognized a protein of ~71 kDa in the β-dystrobrevin preparation (Fig. 6*A*, *β-Db panel*, *β-Db lane*). This staining was blocked by preincubation of the antibody with the antigenic peptide (data not shown). Furthermore, the antibody did not cross-react with the ~60-kDa protein in the α-dystrobrevin-2 preparation that was recognized by pan-Db (Fig. 6*A*, compare *αDb-2 lanes* in the *β-Db* and *pan-Db panels*). Conversely, the α-dystrobrevin-2 antibody (Ab αDB2) labeled ~60-kDa proteins but did not cross-react with β-dystrobrevin (data not shown, but see Fig. 6*B*, *α-DB2 panel*). No reactive proteins were found in control IgG preparations. From these experiments, we conclude that these dystrobrevin antibodies are isoform-specific in immunoaffinity purification and immunoblotting.

In addition to the ~71-kDa form of β-dystrobrevin, an ~40-kDa immunoreactive protein was also specifically enriched in the β-dystrobrevin preparations (Fig. 6*A*, *βDb panel*, *βDb lane*). Although this smaller protein could be a proteolytic fragment, this seems unlikely, since similar proteins were not seen in other preparations (*e.g.* β-dystrobrevins in syntrophin preparations) (Fig. 6*A*, *βDb panel*, *Syn lane*). Instead, the smaller protein may be a product of alternative splicing. However, since we did not observe any mRNAs labeled selectively by probes to the 5′ coding region, additional studies will be needed to confirm this possibility.

Purified α- and β-dystrobrevin complexes appear to be independent. α-Dystrobrevin-2 preparations did not contain detectable levels of either α-dystrobrevin-1 or β-dystrobrevin. Likewise, β-dystrobrevin preparations did not contain α-dystrobrevin-1 or -2. These data argue that, at least in native complexes, the major forms of α-dystrobrevin do not form homotypic associations with β-dystrobrevin.

Association of Dystrobrevins with Syntrophins—The dystrobrevin preparations from brain were also examined for the presence of syntrophins using a pan-specific mAb that recognizes all three syntrophins (40, 41). Compared with rabbit IgG preparations, both α- and β-dystrobrevin specifically copurified with syntrophins (Fig. 6*A*, *Syn panel*). In the reverse experiment, syntrophin preparations contained proteins corresponding to α-dystrobrevin-2 and β-dystrobrevin as well as a major dystrobrevin-immunoreactive protein of the approximate molecular mass of α-dystrobrevin-1 (~80 kDa) (Fig. 6*A*). Thus, both α- and β-dystrobrevin complexes independently associate with syntrophins.

To examine the possibility that dystrobrevin isoforms are differentially associated with syntrophins, we examined syntrophin preparations for dystrobrevin isoforms. For immunoblotting, samples purified from each tissue with the pan-syntrophin antibody were adjusted to contain approximately equal amounts of total syntrophin (as judged by immunoblotting) (Fig. 6*B*, *Syn panel*). Therefore, no conclusions can be drawn from this experiment relating RNA expression to dystrobrevin protein levels. Each tissue examined contained either α-dystrobrevin-2 (skeletal and cardiac muscle) (Fig. 6*B*, *α-Db2 panel*) or β-dystrobrevin (brain, lung, liver, kidney, or testis) (Fig. 6*B*, *βDb panel*) in association with syntrophin but not high levels of both. In several tissues, however, α-dystrobrevin-1 was coexpressed with α-dystrobrevin-2 or β-dystrobrevin (data not shown, see Ref. 14). Thus, in each tissue, the particular dystrobrevin isoform(s) that associates with syntrophins appears to be regulated.

We have previously noted that several mouse tissues express an ~52-kDa form of β1- and β2-syntrophins in addition to the ~60-kDa full-length forms (41). Although these β-syntrophin short forms remain to be characterized, it is noteworthy that their presence coincides with β-dystrobrevin (Fig. 6*B*).

β-Dystrobrevin in Dystrophin Complexes—Recent work indicates that α-dystrobrevin associates with dystrophin in skeletal muscle (17, 41, 52).[2] To examine the possibility that β-dystrobrevin also associates with dystrophin, we immunoisolated β-dystrobrevin from liver, which contains a short form of dystrophin (Dp71) (9) and high levels of β-dystrobrevin but lacks α-dystrobrevin (see Fig. 6*B*). Immunoisolated β-dystrobrevin specifically copurified with Dp71 (Fig. 7*A*). Conversely, anti-dystrophin immunoisolates from liver contained a single dystrobrevin-immunoreactive protein of ~71 kDa. This protein is also recognized by anti-β-dystrobrevin antibodies (Fig. 7*B*). Rabbit IgG-purified control samples were negative. Dystrophin-dystrobrevin complexes isolated with either dystrophin or dystrobrevin antibodies also contained significant amounts of syntrophins (data not shown).

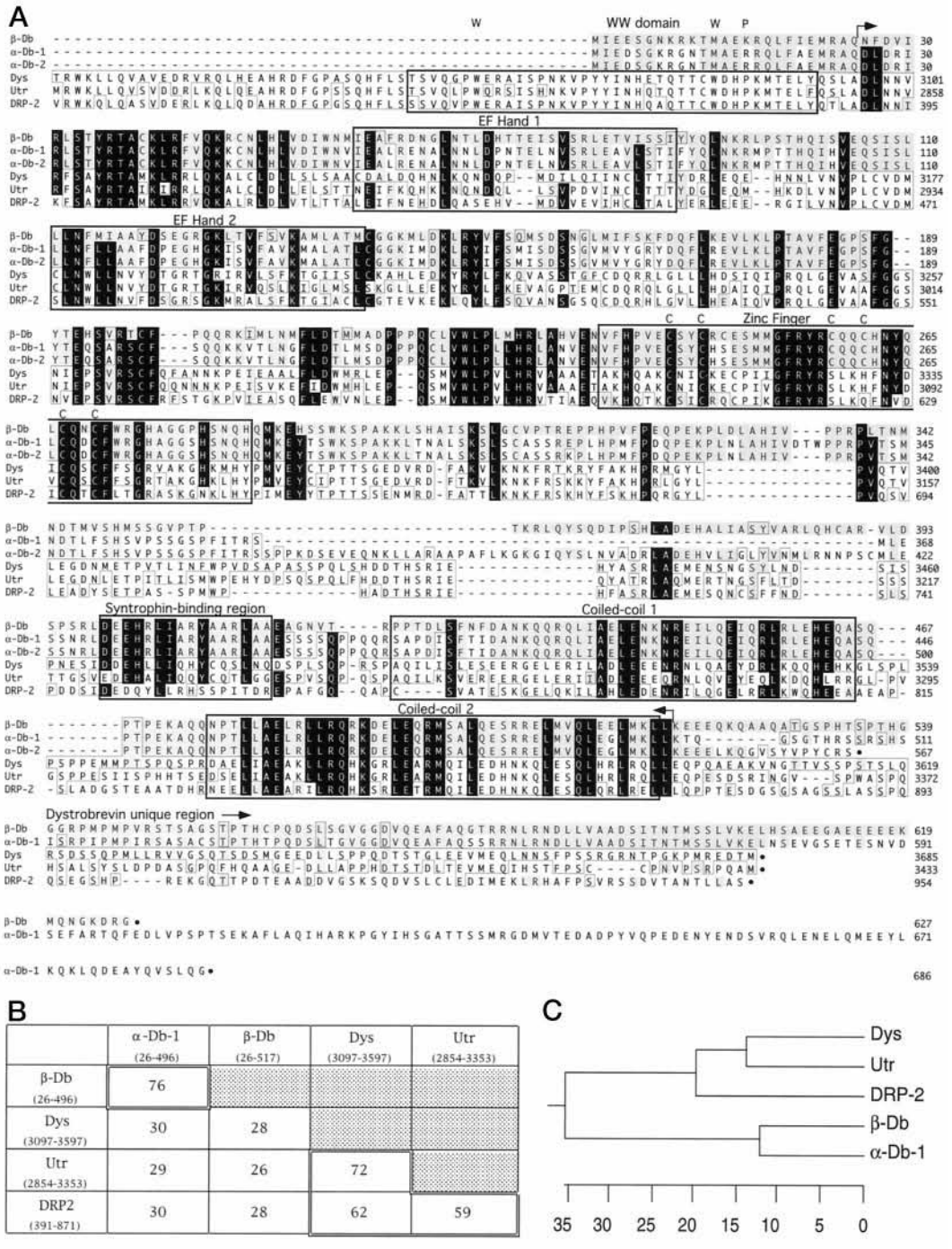

FIG. 5. **Comparison of human β-dystrobrevin with human dystrophin family members.** *A*, the deduced sequence of β-dystrobrevin (*β-Db*) is aligned with that of α-dystrobrevin-1 (*α-Db-1*) (U46744), α-dystrobrevin-2 (*α-Db-2*) (U46745) (15), dystrophin (*Dys*) (M18533) (49), utrophin (*Utr*) (X69086) (16), and DRP2 (U43519) (11). All sequences are human. Sequences of dystrophin, utrophin, and DRP are limited to the cysteine-rich, carboxyl-terminal domain. Residues that are identical in at least four proteins are decorated in *black*. Of the remaining residues, those that are identical to dystrophin are *boxed* (*light*), whereas those that are identical to β-dystrobrevin are *shaded gray*. *Bold boxes* indicate protein domains (see text). Critical residues in the WW domain and the ZZ zinc finger domain are indicated. *B*, identity in the region of highest sequence conservation (denoted by *arrows* in A) was determined by pair-wise comparisons. Data represent percent amino acid identities over the region indicated in parenthesis, as determined with the aid of DNAStar software package. *C*, dendrogram generated from the comparisons in B.

DISCUSSION

Dystrobrevins, a Distinct Subfamily of Dystrophin-related Proteins—We have characterized a fifth member of the dystrophin-related protein family. The new protein, named β-dystrobrevin, is most closely related to the previously identified α-dystrobrevin and more distantly related to dystrophin, utrophin, and DRP2. Thus, two subfamilies of dystrophin-related proteins, the dystrophin family and the dystrobrevin family, can be distinguished based on their primary sequence. Furthermore, we present evidence that dystrophin and β-dystrobrevin are complexed together.

The dystrophin and the dystrobrevin protein families have several features in common, including the coiled-coil structures and the syntrophin binding motif. The coiled-coil structures are highly conserved within each subfamily but are quite divergent across the dystrobrevin and dystrophin subfamilies. Because of

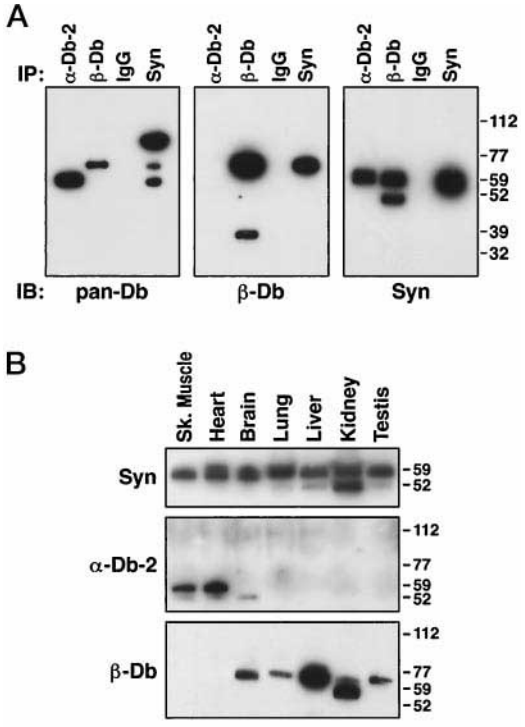

FIG. 6. **Copurification of β-dystrobrevin and syntrophins from brain.** *A*, dystrobrevin, syntrophin, and control samples were immunopurified (*IP*) with Ab αDB2 (*α-DB-2*), Ab βDB344 (*β-DB*), pan-syntrophin mAb SYN1351 (*Syn*), or rabbit IgG from detergent-solubilized brain extracts and subjected to immunoblotting (*IB*). Immunoblotting with pan-dystrobrevin mAb 13H1 (*pan-Db panel*) showed specific enrichment of an ~60-kDa protein in α-dystrobrevin-2 preparations and an ~71-kDa protein in β-dystrobrevin preparations. Syntrophin preparations also contained ~60- and ~71-kDa dystrobrevin-immunoreactive proteins as well an ~80-kDa protein that presumably corresponds to α-dystrobrevin-1. β-Dystrobrevin preparations contained a ~40-kDa protein recognized by Ab βDB344 (*β-Db panel*). Consistent with the idea that this 40-kDa protein is similar to α-dystrobrevin-3, it was not recognized by mAb 13H1, nor did it copurify with syntrophin. Syntrophins were identified in both α- and β-dystrobrevin preparations (*Syn panel*). Representative blots of three experiments are shown. *B*, syntrophin complexes were immunoisolated with mAb SYN1351 from tissue detergent extracts. Sample loadings were adjusted for approximately equal amounts of total syntrophin as judged by immunoblotting (*Syn panel*). Duplicate blots were probed with antibodies αDB2 and βDB344. Molecular mass markers are shown in kDa. *sk.*, skeletal.

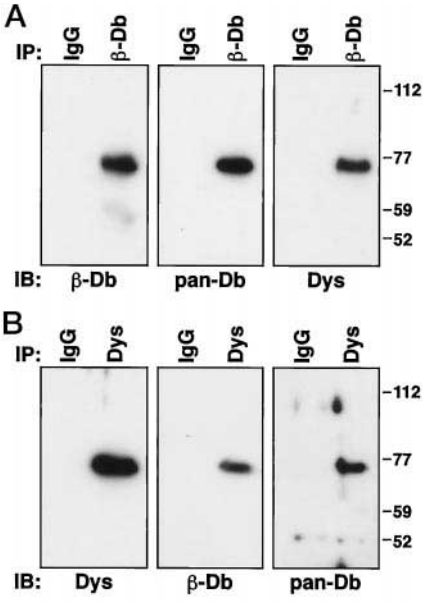

FIG. 7. **Copurification of β-dystrobrevin and the dystrophin short form Dp71 from liver.** *A*, β-dystrobrevin was immunoaffinity-purified from detergent-solubilized extracts of liver, a rich source of β-dystrobrevin. Ab βDB344 preparations (*β-Db*) contained a single dystrobrevin-immunoreactive protein recognized by the β-dystrobrevin antibody (*β-Db panel*, Ab βDB344) and by the pan-dystrobrevin mAb (*pan-Db panel*, mAb DB13H1). A dystrophin-immunoreactive protein of ~71 kDa specifically copurified with β-dystrobrevin (*Dys panel*, MANDRA-1). Control samples prepared with rabbit IgG were negative. *B*, dystrophin protein complexes isolated by immunoprecipitation with mAb MANDRA-1 from detergent-solubilized extracts of liver were specifically enriched in a dystrophin-immunoreactive protein of ~71 kDa, presumably Dp71 (*Dys panel*, Ab DYS3669). Dystrophin preparations also contained a single dystrobrevin-immunoreactive protein recognized by the β-dystrobrevin antibody (*β-Db panel*, Ab βDB344) or by the pan-dystrobrevin mAb (*pan-Db panel*, mAb DB13H1). Control samples prepared with rabbit IgG were negative. Molecular mass markers are shown in kDa.

this high conservation, the coiled-coil regions in dystrophin, utrophin, and DRP2 are likely to bind the same protein(s). Likewise, the coiled-coil regions in dystrobrevins are highly similar to each other but are more distantly related to the dystrophin subfamily. Considerable evidence, including that obtained from colocalization (12), copurification (13, 41), and biochemical association (17) experiments supports the idea that α-dystrobrevin and dystrophin are directly associated. Yeast two-hybrid experiments, in particular, demonstrated that the coiled-coils mediate this interaction.[2] Thus, since α-dystrobrevin binds dystrophin, it is not surprising that we find β-dystrobrevin associated with Dp71, a CRCT dystrophin short form. Taken together with previous results, our findings suggest that dystrophin can associate directly with either α- or β-dystrobrevin via a coiled-coil interaction.

In contrast to these readily detectable associations between dystrophin and dystrobrevins, we were unable to find associations among α- and β-dystrobrevins (Fig. 6) or among dystrophin and utrophin (9, 41). Thus, the two protein subfamilies proposed on the basis of primary sequence appear also to be functionally distinct, at least in their associations with other members of the dystrophin family.

Dystrophin-dystrobrevin complexes may contain two syntrophins, since both dystrophin and dystrobrevin are known to bind syntrophins directly. This prediction of two syntrophins per complex is consistent with previous biochemical studies in which pairs of syntrophin isoforms copurify with dystrophin or utrophin from skeletal muscle (41) and with previous reports of a ~2:1 stoichiometry of syntrophin to dystrophin (53, 54).

Another protein known to associate with dystrophin is β-dystroglycan, a transmembrane protein that in turn links α-dystroglycan to the complex (reviewed in Ref. 55). A large segment of dystrophin is required for high affinity binding of β-dystroglycan, although a core region of 210 amino acids appears to be sufficient for association (52, 56). Included within this core region is a WW domain (48). Conversely, the dystrophin binding site on β-dystroglycan has been narrowed to a 15-amino acid region that includes a consensus WW recognition sequence (48, 56). These studies suggest that binding to β-dystroglycan involves the WW domain of dystrophin, but that additional flanking sequence is required for high affinity binding (52, 56, 57). The WW domain is well conserved in dystrophin, utrophin, and DRP2 but is not found in either of the dystrobrevin isoforms. Thus, the dystrobrevins probably do not contain a high affinity binding site for β-dystroglycan.

Regulation of Dystrophin Complexes—Dystrophin-dystrobrevin complexes are expressed in a wide range of cell types and are likely to be tailored for different membrane specializations. This may be achieved in part by assembling distinct DAP complexes. The dystrophin complex can be dissociated into a three subcomplexes: a cytoplasmic complex containing dystro-

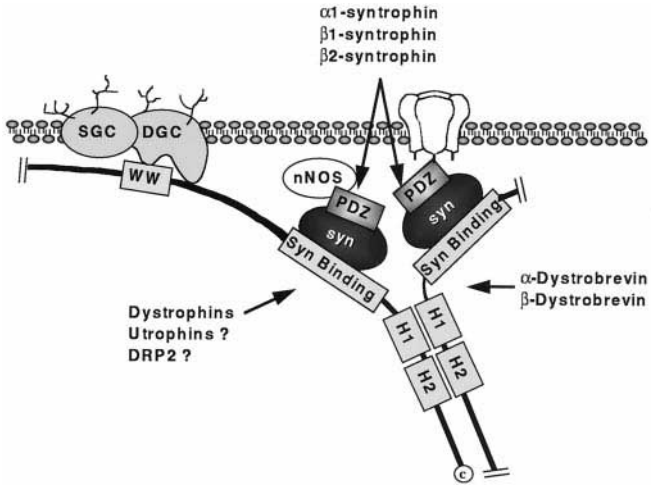

FIG. 8. **A hypothetical model of dystrophin-dystrobrevin-syntrophin complexes.** The dystrophin glycoprotein complex can be dissociated into dystroglycan (*DGC*), sarcoglycan (*SGC*3), and dystrophin-dystrobrevin-syntrophin subcomplexes (58). The region of dystrophin that binds β-dystroglycan includes the WW domain and the adjacent COOH-terminal sequence (52, 56, 57). This β-dystroglycan binding region is absent or poorly conserved in dystrobrevins. Dystrophin and dystrobrevin associate via their coiled-coiled region (17),[2] although the stoichiometry and orientation of this interaction remains to be examined. Here, we identify dystrophins and dystrobrevins as separate subfamilies and propose that each dystrophin family member may associate with each of the dystrobrevins. The association of dystrophins and dystrobrevins results in two syntrophin binding sites in a single complex (41). Although these syntrophin binding sites are not selective *in vitro* (43, 44), native complexes associate with particular syntrophin pairs (41). In skeletal muscle, syntrophin isoforms have been shown to bind nNOS and voltage-gated sodium channels (27, 34). Binding partners for syntrophins in other cell types are unknown. Multiple isoforms of each member of the dystrophin-dystrobrevin-syntrophin subcomplex have been identified. Thus, the particular combination of isoforms of each of these proteins may determine the function of the membrane complex assembled.

phin, dystrobrevin, and syntrophin, and two transmembrane subcomplexes, the sarcoglycans and the dystroglycans (58). Multiple related isoforms of each protein in the cytoplasmic subcomplex have been found to be expressed in a wide range of tissues. In contrast, the sarcoglycans are highly muscle-specific (59), and the dystroglycans are encoded by a single gene (55). Conceivably, products of each dystrophin gene could combine with either α- or β-dystrobrevin and bind any pair of syntrophins, thereby generating an enormous number of different complexes (see Fig. 8). Since syntrophins associate with signaling proteins such as neuronal nitric oxide synthase (34) and voltage-gated sodium channels (27), the regulation of the dystrophin-dystrobrevin-syntrophin subcomplexes is indeed a likely mechanism for directing the assembly of highly specialized membranes.

Within the dystrophin complex, regulation of dystrobrevins may play a particularly important role in determining the proteins recruited to the membrane by dystrophin. Most dystrobrevin products contain a core region (a pair of coiled-coils and a syntrophin-binding region) that is thought to mediate protein-protein interactions essential for assembling native dystrophin complexes. However, the sequences flanking this core region are subject to extensive post-transcriptional and post-translational modifications. For example, α-dystrobrevins-4 and -5, short forms translated from an internal methionine start codon, lack 385 amino acids immediately NH_2-terminal to this core region. Similarly, the region immediately COOH-terminal of the central coiled-coils is subject to alternative splicing. This DUR is composed of ~180 amino acids in full-length α-dystrobrevin-1, whereas in β-dystrobrevin, this region is only ~90 amino acids. In α-dystrobrevins-2 and -5, the DUR is entirely removed by alternative splicing. Finally, the extreme COOH-terminal region of the DUR contains tyrosine phosphorylation sites (13). Thus, the diversity of dystrobrevin products is quite large, suggesting that extensive regulation of dystrobrevins may be important for recruiting different proteins to the membrane via association with the dystrophin complex.

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